Plant Science 243 (2016) 10–22

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

A higher sink competitiveness of the rooting zone and invertases are involved in dark stimulation of adventitious root formation in *Petunia hybrida* cuttings

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ARTICLE INFO

Article history:
Received 3 September 2015
Received in revised form 29 October 2015
Accepted 1 November 2015
Available online 11 November 2015

Keywords:
Photosynthesis
Carbon allocation
Root development
Enzyme activity
Gene expression
Storage

ABSTRACT

The contribution of carbon assimilation and allocation and of invertases to the stimulation of adventitious root formation in response to a dark pre-exposure of petunia cuttings was investigated, considering the rooting zone (stem base) and the shoot apex as competing sinks. Dark exposure had no effect on photosynthesis and dark respiration during the subsequent light period, but promoted dry matter partitioning to the roots. Under darkness, higher activities of cytosolic and vacuolar invertases were maintained in both tissues when compared to cuttings under light. This was partially associated with higher RNA levels of respective genes. However, activity of cell wall invertases and transcript levels of one cell wall invertase isogene increased specifically in the stem base during the first two days after cutting excision under both light and darkness. During five days after excision, RNA accumulation of four invertase genes indicated preferential expression in the stem base compared to the apex. Darkness shifted the balance of expression of one cytosolic and two vacuolar invertase genes towards the stem base. The results indicate that dark exposure before planting enhances the carbon sink competitiveness of the rooting zone and that expression and activity of invertases contribute to the shift in carbon allocation.

1. Introduction

Petunia hybrida is one of the economically most important ornamental plant species and a great proportion of plants are generated by vegetative propagation. The key developmental process in determining its successful propagation is adventitious root (AR) formation in the stem base of leafy shoot tip cuttings. Stock plants for cutting production of petunia and other plant species are cultivated in tropical regions or low latitude sites. After harvesting the cuttings, they are densely packed and transported to rooting stations in the main business markets of Central Europe and the USA [1,2]. There, cuttings are rooted under low irradiation during winter to provide plants to the consumers in spring and early summer. The transport period is a necessary step within the production chain of petunia young plants and usually takes place in the dark. Additionally, cuttings are intermediately stored usually in the dark at reduced temperatures for collecting cuttings before rooting to cope with the temporarily high demands for young plants of certain cultivars [3]. Low temperature storage slows down plant metabolism and extends shelf life of cuttings [4–6]. However, the storage potential of the plant is dependent on its genotype. *Pelargonium* has been repeatedly described as storage-sensitive genus responding to dark exposure with senescence of cuttings or insufficient AR formation thereafter [7–10]. A good storage tolerance was found for carnation [11–13], chrysanthemum [14] and also for petunia [2]. Dark exposure of cuttings of the petunia cv. 'Mitchell' strongly increased the intensity of subsequent AR formation under light.

AR formation in cuttings is a multistage developmental process, which is controlled by plant hormones particularly auxin [15]

Abbreviations: AR, adventitious root; cw, cell wall; cyt, cytosolic; DM, dry matter; dpe, days post excision; dpin, days post insertion; FW, fresh weight; INV, invertase; P_N, net photosynthesis; PPFD, photosynthetic photon flux density; R_D, dark respiration; vac, vacuolar.

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http://dx.doi.org/10.1016/j.plantsci.2015.11.001

and also depends on the availability of diverse resources in the rooting zone. There, carbohydrates are particularly important for providing the energy and carbon skeletons to power and feed the cell differentiation, development and growth of new roots [16–18]. In addition, carbohydrates may directly control root development via modulation of gene expression and interaction with plant hormones [13,18–20]. Interestingly, the level of sugars particularly of sucrose and the partitioning between sucrose and starch in cutting leaves seem to provide important bottlenecks for AR formation in the stem base [9,10,14,21]. This stays in accordance with the outstanding role of sucrose as major carbohydrate fraction exported from source tissues and translocated to the diverse utilization and storage sinks in the plant [22,23]. Carbohydrate levels in the cutting tissues are affected by the initial carbohydrate reserves [9,24] and the current carbon assimilation during rooting by photosynthetic activity [10,25–28].

Diminishing the carbon source is only one factor which might limit AR formation. Differences in the strength among the diverse carbohydrate sinks together with the activity of source to sink pathways additionally determine the channelling of assimilates to the different utilization and storage sinks in a plant [22,29]. Invertases are important molecular drivers of sink strength, since they reduce the sucrose pool by converting it into glucose and fructose, which are further channelled into the metabolic pathways, and thereby regulate utilization and storage of organic carbon. The activities of invertases indirectly modulate gene expression via modifying the level of hexoses that regulate cell cycle and cell division programs. In this context, invertases are already proven to be vital for the establishment of "young sinks" such as flowers and fruits [30]. Among the compartment-specific types of vacuolar invertases (INVvac), cytosolic invertases (INVcyt) and cell wall invertases (INVcw), the latter are considered to have an outstanding role in sink activity via modifying phloem unloading particularly in those sink tissues that undergo cell division and elongation [29].

In petunia cuttings, the establishment of the new sink in the rooting zone is an early metabolic key event involved in AR formation at standard conditions under diurnal light [31,32]. During the "sink establishment" phase, increased INVcw activity obviously contributes to an apoplastic unloading of sucrose, while simultaneous depletion of sugars indicates carbohydrate utilization in the rooting zone. Recent studies of the response of cuttings of *P. hybrida* 'Mitchell' to dark exposure revealed a decrease of sugar levels in fully developed source leaves and the stem base during the dark period of seven days, while formation of root meristemoids already started [2]. Interestingly, strong enhancement of AR development during the subsequent light period was associated with higher accumulation of carbohydrates particularly in the stem base during the first three days post insertion (planting) of dark pre-exposed cuttings when compared to cuttings which did not experience dark exposure before planting [2]. The increased carbohydrate levels could be the result of increased photosynthesis caused by possible feed-forward control of carbohydrate depletion during darkness [33–35].

The first objective of this study was to evaluate, whether the enhanced carbohydrate levels and root formation in the stem base of dark pre-exposed cuttings is the outcome of a higher source activity (net carbon assimilation) or of higher sink strength in the rooting zone. This question was addressed by the analysis of CO_2 gas exchange and of dry matter production and allocation between the shoot and root. Based on the results, we followed the hypothesis that organ-specific activation of invertases is involved in dark-stimulated dry matter allocation towards the rooting zone. Therefore, we monitored enzyme activities and RNA accumulation levels in the stem base and in the shoot apex, which constitutes an important utilization sink competing with the rooting zone.

2. Material and methods

2.1. Plant material, growth conditions and treatment of cuttings

Seeds of *P. hybrida* cv. 'Mitchell' were sterilised and germinated and stock plants were established as described by Klopotek et al. [2]. Eighty potted stock plants (fertilised peat substrate: Einheitserde Typ ED-73 with Optifer, Patzer, Sinntal-Jossa, Germany) were placed in the greenhouse and fertilised repeatedly with Hakaphos special (16% N, 8% P_2O_5, 22% K_2O, 3% MgO + micronutrients, COMPO GmbH Münster, Germany) following commercial horticultural practice. Temperature and light in the greenhouse were controlled as described [2]. The experiments were performed about three months after the germination of the stock plants. Leafy cuttings were excised from the stock plants four hours after commencing the light period. Cuttings were obtained by using shoot tips containing four to five leaves (Fig. 1), leaving two nodes of the shoot on the plant [36].

The general experimental set-up is illustrated in Fig. 1. Control cuttings, which were planted and exposed to diurnal light conditions immediately after excision, were compared with cuttings, which first experienced a dark exposure for 7 days and thereafter were planted and exposed to diurnal light conditions. In the results part, the term "dark" is used, when the dark-treated cuttings are under the dark conditions, the term "post-dark" or "dark pre-exposed" is used for the dark-treated cuttings when they have been planted after the dark exposure and are cultivated under same diurnal light conditions as the immediately planted control cuttings. Two time-scales were used (Fig. 1). Days post excision (dpe) refer to the time when cuttings were excised. Days post insertion (dpin) refer to the time when cuttings were inserted (planted) into the rooting substrate perlite and exposed to diurnal light conditions. The presented data involved six experiments (one on photosynthesis, two on dry matter partitioning, two on invertase activity, one on RNA accumulation of invertase genes) focussing on different specific phases of this experimental set-up.

Since the promotive influence of the dark exposure on AR formation was evident with both temperatures of 10 °C and 20 °C but leaf yellowing and drying was observed with 20 °C [2], the dark exposure was conducted at 10 °C. For a seven day dark exposure, cuttings were put in non-perforated bags in a cardboard box that was stored in a dark cabinet at 10 °C.

For rooting, 16–20 cuttings per tray were inserted in perlite Perligran A with a particle size of 0–6 mm and a pH of

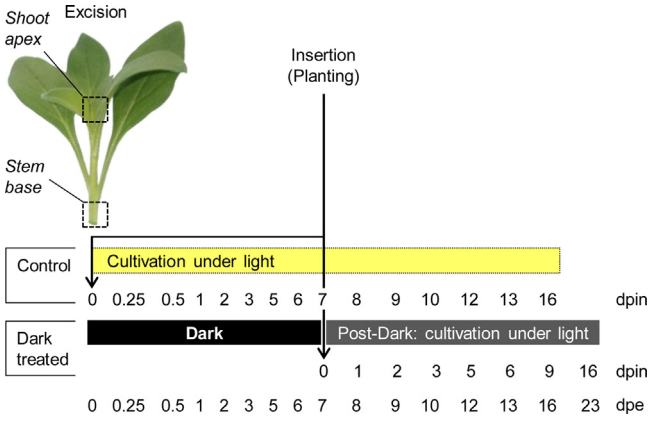

Fig. 1. Schematic presentation of the experimental set-up applied to study the influence of dark treatment of cuttings on photosynthesis, dry matter allocation between shoot and roots and invertase activities and RNA accumulation in shoot apex and stem base. Dpe indicate days post excision, dpin indicate days post insertion (planting). The time points considered depended on the particular focus of the experiment.

7.0 (Knauf Perlite GmbH, Dortmund, Germany) in plastic trays (46 cm × 28 cm × 5 cm, not sub-divided in cells), covered with light-transparent plastic hoods and exposed to diurnal light conditions in a growth chamber (settings of temperature: 22/20 °C day/night, humidity: 85/60% day/night, light: 10 h day length). PPFD at plant level was adjusted to 100 μmol m^{-2} s^{-1}. Non-treated cuttings (control) were immediately inserted in rooting trays and exposed to growth chamber conditions. The dark pre-exposed cuttings were treated in the same way after the seven day dark period. The cuttings were irrigated daily with tap water. The trays covered with plastic were also used as cuvettes for the CO_2 gas exchange measurements.

2.2. Measurement of CO_2 gas exchange

After insertion of petunia cuttings in perlite filled rooting trays, continuous measurement of net CO_2 gas exchange rates of whole cuttings (control versus dark pre-exposed cuttings) were conducted in the growth chamber using an open chamber system according to Klopotek et al. [36]. The temperature in the cuvettes was 22 °C during the 10-h photoperiod and 20 °C during the 14-h dark period. The PPFD under the plastic hood averaged 100 μmol m^{-2} s^{-1}, the CO_2 concentration in the growth chamber was adjusted to achieve a CO_2 concentration of approximately 400 ppm under the plastic hood. The relative humidity of the air in the cuvettes was approximately 85%. The CO_2 gas exchange rate during the photoperiod was interpreted as net photosynthesis (P_N, μmol m^{-2} s^{-1}) and the positive value of the gas exchange rate during the dark phase was taken as (dark) respiration (R_D, μmol m^{-2} s^{-1}) [36]. The leaf area of 64 cuttings per treatment was measured destructively at 0 dpin and 7 dpin using a leaf area meter LI-3100 (LI-COR Inc., Lincoln, U.S.A.). Based on these data, the leaf areas during the experimental period were estimated by linear interpolation.

2.3. Analysis of dry matter production

The starting shoot length and fresh weight (FW_E) of the evaluated cuttings was determined at harvest before exposure to the dark and/or rooting conditions. For non-destructive determination of the starting dry matter of the evaluated cuttings (DM_E), the percentage of dry matter determined in parallel batches of harvested cuttings was taken into account. Therefore, the fresh weight of parallel cuttings (FW_P) was measured and the dry matter (DM_P) was determined after drying in a ventilated oven at 80 °C for two days. The starting dry matter of the evaluated cuttings was calculated as follows:

$$DM_E = \frac{(FW_E \times DM_P)}{FW_P}$$

After different periods of dark exposure and rooting under diurnal light, shoot length and shoot and root dry matter of treated cuttings were determined. Dry matter production was calculated as increase in dry matter above DM_E.

2.4. Extraction and activity measurement of invertases

In the first experiment on invertase activity, samples of 0.5 cm stem base and of shoot apex (including adjacent small leaves, total length ca. 2 cm) of control and dark exposed cuttings were taken at 0, 0.25, 0.5, 1, 2, 3, and 5 dpe (=dpin in case of control cuttings) and of cuttings, which had been dark exposed for 7 days, additionally at 0, 1, 2, 3 and 5 dpin during the subsequent rooting period under diurnal light in the growth chamber. An additional experiment and measurement of INVcw activity was carried out where samples of the apex and the stem base of control and dark exposed cuttings were taken at 0, 1 and 2 dpe. Extraction of all samples was performed according to Zrenner et al. [37] with minor modifications according to Akhami et al. [31].

The activity of INVvac and INVcyt was assayed with the samples supernatant as described by Zrenner et al. [37]. After enzyme extraction, the remaining pellet was used for the measurement of INVcw [31]. Invertase activities were determined via enzymatic assay in microplates by measuring the extinction changes of NADH + H^+ at a wavelength of 340 nm. Dark exposure significantly affected the protein level in the tissues (data not shown). To avoid artefacts resulting from dark induced changes of levels of other proteins such as Rubisco [38], the invertase activity data was related to the fresh mass.

2.5. RNA extraction and analyses of invertase gene RNA levels

RNA was extracted from pooled shoot apices and stem bases of the cuttings using the RNeasy Mini Kit (Qiagen, Hilden, Germany) followed by DNase I treatment (Qiagen) as described by Ahkami et al. [31]. 4 μg total RNA were reverse transcribed with oligo(dT) and the M-MLV reverse transcriptase following the protocol of the supplier (Promega, Mannheim, Germany). Genes for calibration were tested according to Mallona et al. [39] showing that *PhTEF1α* and *PhRAN1* were most suitable for RNA accumulation analysis of petunia cuttings (data not shown). Primers for the real-time PCR for the invertase genes were designed using the DNAStar Primer Select software (GATC Biotech, Konstanz, Germany) and are shown in Table 1. Sequences were derived from the list of genes published by Breuillin et al. [40]. Quantitative real-time polymerase chain reaction (PCR) was carried out using the 7500 Fast real-time PCR System (Applied Biosystems, Darmstadt, Germany) with the following temperature program: 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 15 s at 95 °C and 1 min the annealing temperature (Table 1). PCR reactions were performed with three biological repeats each with three technical repetitions. Relative RNA accumulation values were cal-

Table 1
Primer sequences for the invertase genes (gene identifiers according to Breuillin et al. [40]) and annealing temperatures for real-time PCR.

Gene names and identifiers	Primer sequences	Annealing temperature
PhINVcyt1 (cn7412)	For: AAGCAGGCCTGGAAGTTGTTG Rev: AGGGCAGATGGAATAAAGTC	56 °C
PhINVcyt2 (GO_drpoolB-CL1857Contig2)	For: GATCGCAGAATGGGTGTAT Rev: CCTGAAGGCGTTTAGTTATT	54 °C
PhINVvac1 (cn9152)	For: TCAGTGGCCCGTAGAAGA Rev: AATTGAGCCGGGTTGAAGAT	56 °C
PhINVvac2 (cn6751)	For: CCCGGAGTTGGATGTTGGTATC Rev: TGGAGATCAGCAGCTTCAGTGTC	54 °C
PhINVcw1 (cn8827)	For: GAGTTTATCCTGCTTTGGCTATC Rev: GTTATTCGTCCTTGGCTTCATTG	59 °C
PhINVcw2 (cn8044)	For: AAATCATCATGCTCTCACCTC Rev: TCCAAGTTAAATAAATCAATGC	56 °C

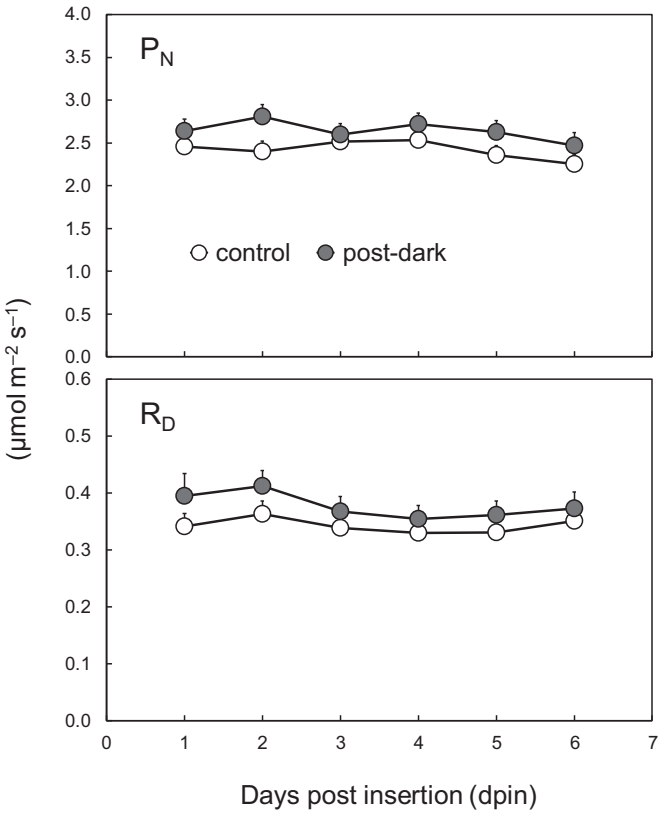

Fig. 2. Net photosynthesis (P_N) and dark respiration (R_D) of petunia cuttings during rooting as affected by dark pre-exposure of cuttings. Control: cuttings which had been planted (inserted into the rooting substrate) after excision. Post-dark: cuttings which had been dark exposed for 7 days after excision, before planting (see scheme in Fig. 1). Mean values and SEs per day. No significant differences were recorded between the two treatments (*t*-test, $P < 0.05$, $n = 8$, each consisting of 16 cuttings).

culated as $2^{\Delta Ct}$ with ΔCt as the difference of the values obtained for the invertase genes and the geometric mean of the two reference genes.

2.6. Statistics

All data are given as means and standard errors (SE). Statistical analyses were performed using STATISTICA© 263 6.1. software (StatSoft). Data of CO_2 gas exchange, dry matter production, enzyme activity and RNA accumulation analyses were compared between dark treated and control cuttings by use of the *t*-test at significance level $P \leq 0.05$. The same *t*-test was used for testing significant up- or down-regulation of invertase genes compared to 0 dpe and 0 dpin.

3. Results

3.1. CO_2 gas exchange

Considering the stimulation of root development and accumulation of higher levels of carbohydrates in the rooting zone during the first days after dark exposure [2], the first experiment addressed the question, whether dark pre-exposure of cuttings enhances net photosynthesis after planting. CO_2 gas exchange of whole cuttings was monitored for 6 days under diurnal light conditions. Control cuttings, which were planted immediately after excision from donor plants, were compared with cuttings which had been dark exposed for seven days after severance before planting. Dark pre-exposure of petunia cuttings had no effect on CO_2 gas exchange (Fig. 2). Neither P_N nor R_D rates per leaf area of dark pre-exposed cuttings showed significant differences compared to the control during the whole course of the experiment. In contrast, the CO_2 gas exchange rates of the control and the dark pre-exposed cuttings remained at initial levels without distinctive alterations during the monitoring period. Similar results were determined when P_N and R_D were calculated per cutting as reference parameter (data not shown).

3.2. Dry matter production and allocation between shoot and root

To elucidate whether carbon utilisation within the cutting is altered by dark exposure, the allocation of dry matter between shoot and root was studied. At first we focused on temporary dark (0–7 dpe) and post-dark (8–16 dpe) effects compared to continuous diurnal light (0–16 dpe) during a same period of 16 dpe, which has been found to be sufficient for attaining intensive rooting of dark treated cuttings. Shoot dry matter of control cuttings increased significantly during the first 7 dpe under light whilst shoot dry matter remained unchanged within the same period under dark (Fig. 3A). During the following period of rooting under same light conditions from 7 to 16 dpe, control and the dark pre-exposed cuttings presented similar shoot dry matter production. Considering the overall time course of 16 days after excision, the dark pre-exposed cuttings produced only half of shoot dry matter as control cuttings. This resulted from the lag of shoot dry matter production during the dark phase. A similar relationship was found for the shoot length. During the period of 16 days after excision, shoot length of control cuttings increased by 0.94 ± 0.11 cm, which was significantly higher than shoot length increment of 0.44 ± 0.15 cm for the dark exposed cuttings ($P \leq 0.01$, $n = 4$, each with 10 cuttings).

Contrasting to the strong increase in shoot dry matter (Fig. 3A), control cuttings did not produce any roots during the first 7 days under light. In consequence, root dry matter remained at zero as it was the case for the cuttings which were kept in the dark (Fig. 3C). Root dry matter production started thereafter in cuttings of both treatments. Considering the overall period of 16 days after severance, dark pre-exposed cuttings revealed a higher and more stable root dry matter production than the controls, even though the difference was not statistically significant due to the high variation of data of the controls (Fig. 3C). However, the root dry matter of dark-treated cuttings was the outcome of only 9 days of diurnal light after excision instead of 16 days of diurnal light for the control cuttings. Nevertheless, calculation of the percentage of root dry matter production in total dry matter production (root + shoot) over the period of 0–16 dpe reveals a significantly higher value of about 8% for dark pre-exposed cuttings compared to the controls with about 3% (Fig. 3C). This indicates that during the same time period after excision, temporarily dark-treated cuttings allocated a higher proportion of dry matter towards the roots than cuttings which were planted and exposed to light immediately.

The effect of dark pre-exposure on carbon allocation became even stronger, when control and previously dark exposed cuttings experienced the same period of diurnal light after planting. During a period of 9 days after planting, total dry matter production of control cuttings (117 ± 4 mg) was not statistically different from the dry matter production of dark pre-exposed cuttings (124 ± 2 mg), which is consistent with the same photosynthetic rates determined for both treatments. However, shoot dry matter production over the same period indicated a slightly lower (not significant) value for the dark pre-exposed compared to the control cuttings and this effect was statistically significant after a longer cultivation period of 16 days (Fig. 3B). In contrast, dry matter production of roots, which was generally low compared to the shoot, was significantly enhanced by dark pre-exposure at 9 dpin already (Fig. 3D). As a result, the allocation of dry matter toward the roots was raised by a factor of 12 (12% of total dry matter for dark pre-treated cuttings versus 1%

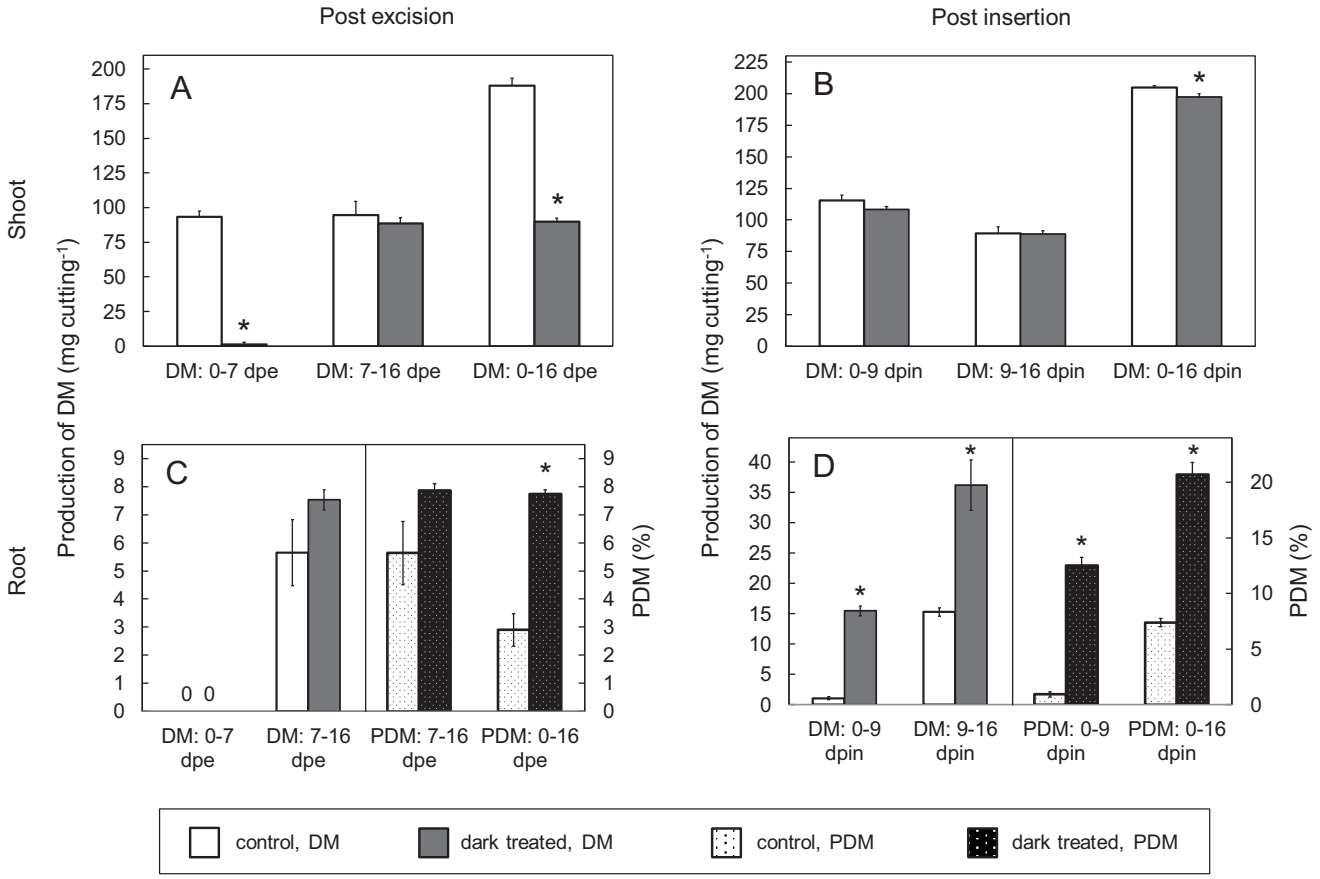

Fig. 3. Dry matter (DM) production of petunia cuttings as affected by light conditions (illustrated in Fig. 1). The production of DM of the shoot (A, B) and of the roots (C, D) and the percentage of root DM in total DM production (PDM in C, D) were determined over different periods after excision (dpe) of cuttings (A, C) and after insertion/planting (dpin) of cuttings (B, D). Mean values and SEs are presented. Asterisks indicate a significant effect of dark storage for the specified period (*t*-test, $P < 0.05$, $n = 4$, each consisting of 20 cuttings). For the *t*-test, the percentage values of PDM were arcus-sinus-root-transformed.

of total dry matter for the control cuttings). When the cultivation period was extended to 16 days, these differences were essentially maintained. Monitoring dry matter at earlier time points in another experiment revealed that stimulation of root dry matter production by dark pre-exposure does not become apparent until 3 dpin (0 mg root dry matter for both treatments at 3 dpin, data not shown).

3.3. Activity of invertases

Our finding that enhanced AR formation in dark pre-exposed cuttings involves enhanced dry matter allocation to the rooting zone under the subsequent light period suggested an important role of sink activity. Considering that invertase genes are strongly regulated during AR formation in petunia cuttings [32], while INVcw probably contributes to the establishment of the new sink in the rooting zone [31], we studied the influence of dark exposure on the activities of INVcw, INVcyt and INVvac. We monitored the activities in the shoot apex and the stem base, since both organs constitute the two competing growth sinks in a shoot tip cutting. Moreover, the current influence of dark treatment and the conditioning effect of the dark treatment on the subsequent rooting under light were evaluated. For this, three types of cuttings were compared: (i) control cuttings subjected to rooting conditions immediately after excision (control), (ii) cuttings that experienced immediate dark storage after excision (dark) and, (iii) cuttings subjected to dark treatment for seven days before planting and then exposed to same rooting conditions as the control (post-dark). Invertase activities were monitored over a period of 5 dpe in the control and dark-treated cuttings and over the period of 5 dpin for the previously stored cuttings. For those cuttings, 0 dpin correspond to 7 dpe, whereas in the case of control cuttings, the dpe and dpin scales are identical (Fig. 1).

Activities of INVcyt and INVvac were on similar levels in both organs (Fig. 4A, B, E, F). They decreased during 5 dpe in the control cuttings, but were maintained on higher levels in both organs under the condition of dark storage. This effect was most pronounced during the period between 0.5 until 2 dpe. Nevertheless, when dark treated cuttings were planted for rooting after 7 days of storage they revealed significantly lower activities of INVcyt and INVvac when compared to control cuttings at the time of excision from the donor plants and planting ("post-dark" versus "control" cuttings at 0 dpin in Fig. 4). During the subsequent rooting under diurnal light, control and post-dark cuttings revealed similar activities of INVvac in both organs and of INVcyt in the apex, whereas significantly lower activities of INVcyt were found in the stem base of post-dark cuttings compared to the control (Fig. 4A, B, E, F).

In the same experiment, the activity of INVcw in the apex remained on similar levels during 5 dpe, for both control and dark exposed cuttings (Fig. 4C). In contrast, INVcw activity in the stem base of the controls increased during the 5 days of rooting under light and from 3 dpe onwards reached significantly higher levels compared to the dark exposed cuttings (Fig. 4G). Interestingly, the dark incubated cuttings revealed a transient increase of INVcw activity, at 1 dpe reaching a level similar to the control cuttings. Considering the large variability of the INVcw data in that experiment and that INVcws are particularly important during the first days after cutting excision, a replicative experiment was conducted focusing on the period of 0 until 2 dpe but involving a higher

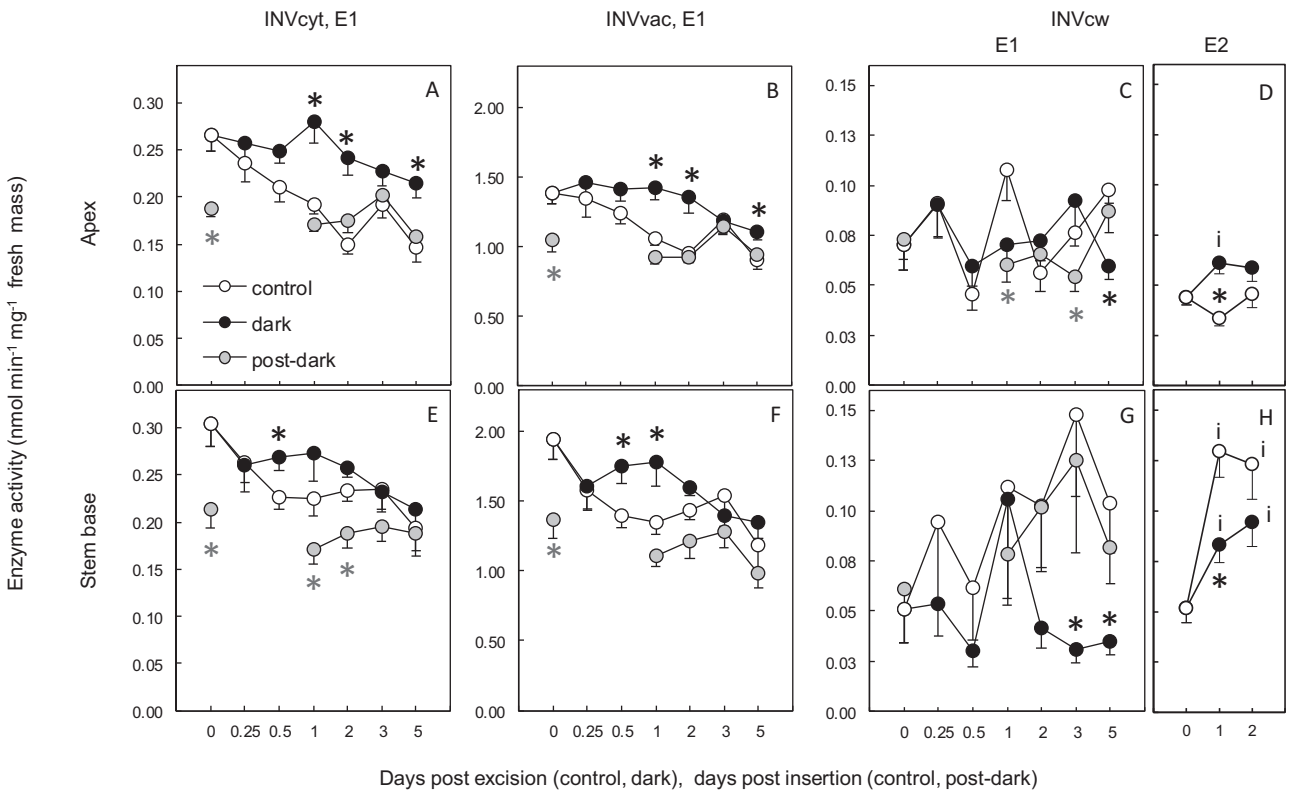

Fig. 4. Activities of cytosolic invertase (INVcyt in A, E), vacuolar invertase (INVvac in B, F) and cell wall invertase (INWcw in C, D, G, H) in the shoot apex (A–D) and the stem base (E–H) of petunia cuttings as affected by light conditions (illustrated in Fig. 1). E1: experiment considering the period of 0–5 dpe/dpin. E2: experiment focusing on 0–2 dpe. Mean values and SEs are presented. Asterisks in black or grey indicate significant differences between the control and the dark or post-dark treatment, respectively, for the indicated time point. i indicates significant higher activity compared to 0 dpe (*t*-test, $P < 0.05$, $n = 8$ (E1), $n = 12$ (E2), each sample from two individual cuttings).

number of biological replicates. This experiment confirmed a significant increase of INVcw activity in the stem base after 1 dpe under both, dark exposure and light conditions, whereas the activity in the shoot apex was again maintained on a similar low level for both treatments (Fig. 4D, H). In this experiment, INVcw activity in the stem base even further increased during the next day of dark exposure to reach a similar level as found for the control cuttings. Nevertheless, all data indicate that dark exposure of cuttings antagonizes a decrease in activities of INVcyt and INVvac in both sink tissues but allows for an early selective rise of INVcw activity in the rooting zone similar to the rise observed under rooting conditions under diurnal light.

3.4. RNA accumulation of genes coding for invertases

To elucidate, whether the observed responses of invertase activity are regulated at transcript level, we analysed RNA accumulation of the corresponding genes in the stem base and the apex by quantitative Real Time PCR. For each type of invertases, RNA levels of two representative genes were measured under control, dark and post-dark conditions. The heatmap in Fig. 5 summarizes the statistics of regulation of the six genes in the two organs over the period of 5 dpe as compared to 0 dpe in the case of control and dark exposed cuttings and over the period of 5 dpin as compared to 0 dpin in case of control and post-dark cuttings. It further shows whether there was a current effect (dark) or downstream effect of dark exposure (post-dark) on RNA accumulation compared to controls. Fig. 6 illustrates the time course of the RNA levels of the respective transcripts.

When compared to 0 dpe, RNA levels of the INVcyt gene *PhINVcyt1* were in both organs of the control cuttings mainly reduced during the 5 dpe period (Fig. 5). Current dark exposure mitigated the down-regulation of *PhINVcyt1* as reflected by significantly higher RNA accumulation levels when compared to control cuttings. This effect was observed more frequently and was also more pronounced in the stem base than in the apex (Fig. 5), with the strongest difference to the control observed at 3 dpe (Fig. 6D). RNA accumulation of the other INVcyt gene *PhINVcyt2* was much higher in the stem base than in the apex and there was strongly enhanced until 3 dpe under both control and dark conditions, while lower levels were found for the dark exposed cuttings (Figs. 5 and 6G, J). After dark exposed cuttings were planted and exposed to diurnal light, RNA levels of the *PhINVcyt1* and *PhINVcyt2* gene were mostly lower compared to the immediately planted control cuttings (Figs. 5 and 6A, D, G, J).

RNA accumulation of the INVvac gene *PhINVvac1* in the apex remained on similar levels after excision of cuttings (Figs. 5 and 6B). However, the same gene was significantly down-regulated in the stem base under control conditions. This down-regulation was mitigated under current darkness resulting in significantly higher RNA accumulation levels for the dark exposed cuttings at 1 and 5 dpe (Figs. 5 and 6E). In addition, after planting of dark exposed cuttings, the RNA level in the stem base accumulated until 1 dpin to a significantly higher value compared to the immediately planted control cuttings (Figs. 5 and 6E). Furthermore, during the period of 0.25 until 1 dpe, dark exposure reduced the RNA levels of *PhINVvac2* in the apex but increased the respective levels in the stem base compared to control conditions (Fig. 5). Thereafter, the RNA of *PhINVvac2* accumulated until 3 dpe in both the apex and the stem base of control and dark exposed cuttings (Fig. 5), while in the stem base same high levels were reached for control and dark exposed cuttings (Fig. 6K)

After cutting excision, RNA levels of the INVcw gene *PhINVcw1* accumulated until 3 dpe in both tissues. However, generally higher levels were attained in the stem base than in the apex

Gene	Organ	Treatment	dpe (control, dark), dpin (control, post-dark)						
			0	0.25	0.5	1	2	3	5
PhINVcyt1	A	Control							
	A	Dark		+	+		–	+	+
	A	Post-dark					–	–	–
	B	Control							
	B	Dark		+	+	+	–	+	+
	B	Post-dark	–				–		
PhINVcyt2	A	Control							
	A	Dark							
	A	Post-dark					–	–	
	B	Control							
	B	Dark			–	–	–	–	–
	B	Post-dark	–			–	–	–	
PhINVvac1	A	Control							
	A	Dark		–	–	–	–	–	
	A	Post-dark				+	–	+	+
	B	Control							
	B	Dark				+			+
	B	Post-dark	–			+	–		+
PhINVvac2	A	Control							
	A	Dark		–	–	–	–	–	–
	A	Post-dark				–	–	–	–
	B	Control							
	B	Dark		+	+	+			
	B	Post-dark	–				–	–	
PhINVcw1	A	Control							
	A	Dark		–	–		–	–	–
	A	Post-dark				–	–	–	
	B	Control							
	B	Dark		+			–	–	–
	B	Post-dark	–			–	–	–	
PhINVcw2	A	Control							
	A	Dark				–		–	
	A	Post-dark	+			–		–	+
	B	Control							
	B	Dark		–	–	–	–	–	–
	B	Post-dark				–	–	–	

	not determined
	up-regulated compared to 0 dpe (dpin)
	down-regulated compared to 0 dpe (dpin)
	not different to 0 dpe (dpin)
+	higher expression compared with Control
–	lower expression compared with Control
A	apex
B	stem base

Fig. 5. Influence of cutting excision and light conditions on RNA accumulation of six genes coding for cytosolic invertases (INVcyt), vacuolar invertases (INVvac), and cell wall invertases (INVcw) in the apex (A) and the stem base (B) of petunia cuttings (treatments are illustrated in Fig. 1). Green and red squares indicate significant down-regulation and up-regulation compared to 0 dpe (dpin), respectively. Plus (+) and minus (−) indicate significantly higher and lower RNA accumulation compared to the control (*t*-test, $P < 0.05$, $n = 3$, each sample from 6 to 9 individual cuttings).

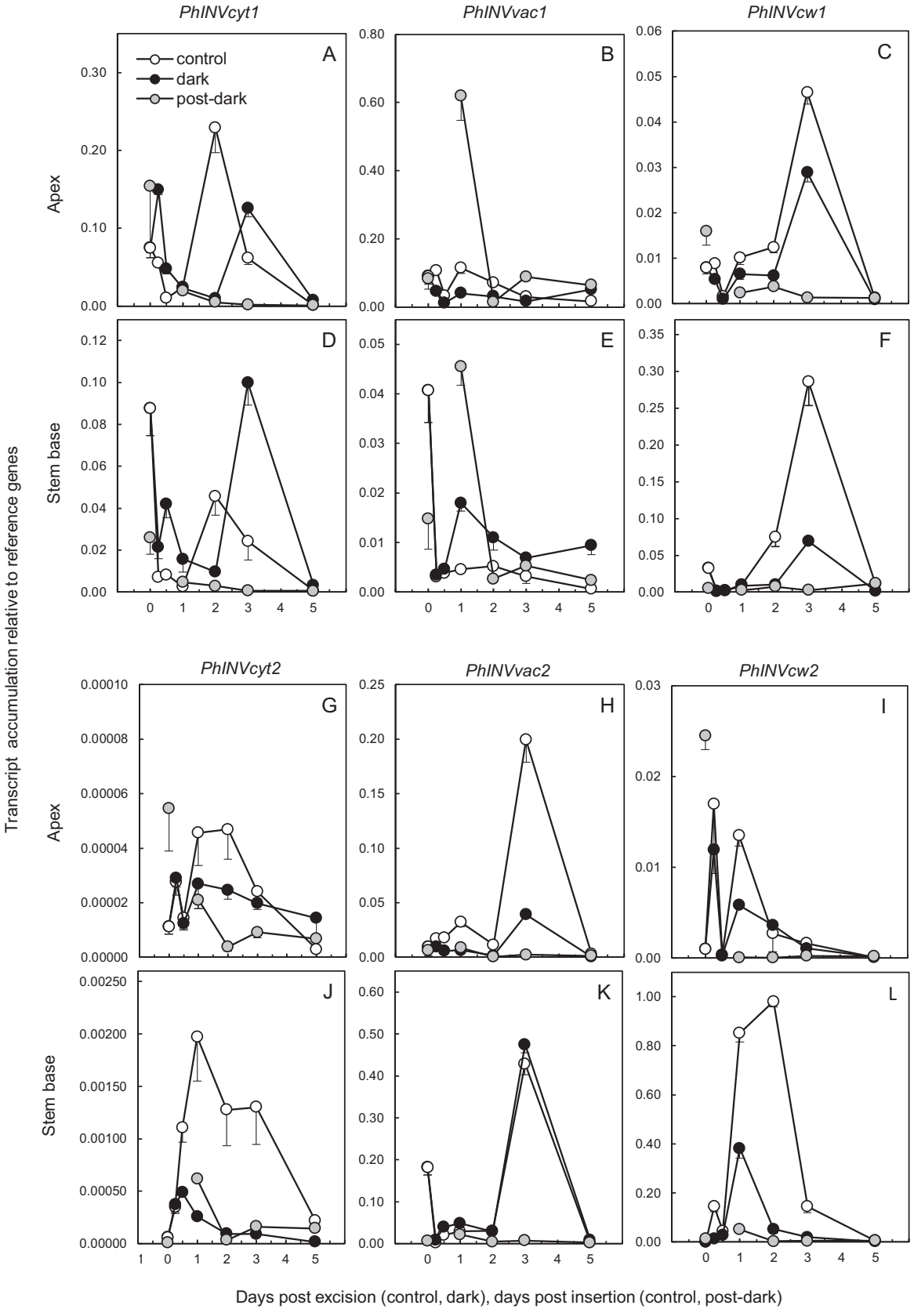

Fig. 6. RNA accumulation levels of genes coding for invertases in petunia cuttings during a period of 5 days post excision and post insertion as affected by light conditions (illustrated in Fig. 1). Mean values and SEs of RNA accumulation levels of two cytosolic (A, D: *PhINVcyt1*; G, J: *PhINVcyt2*), two vacuolar (B, E: *PhINVvac1*; H, K: *PhINVvac2*) and two cell wall invertases (C, F: *PhINVcw1*; I, L: *PhINVcw2*) in the apex (A–C, G–I) and the stem base (D–F, J–L) as related to reference genes are presented ($n = 3$, each sample from 6 to 9 individual cuttings, statistical comparisons are presented in Fig. 5).

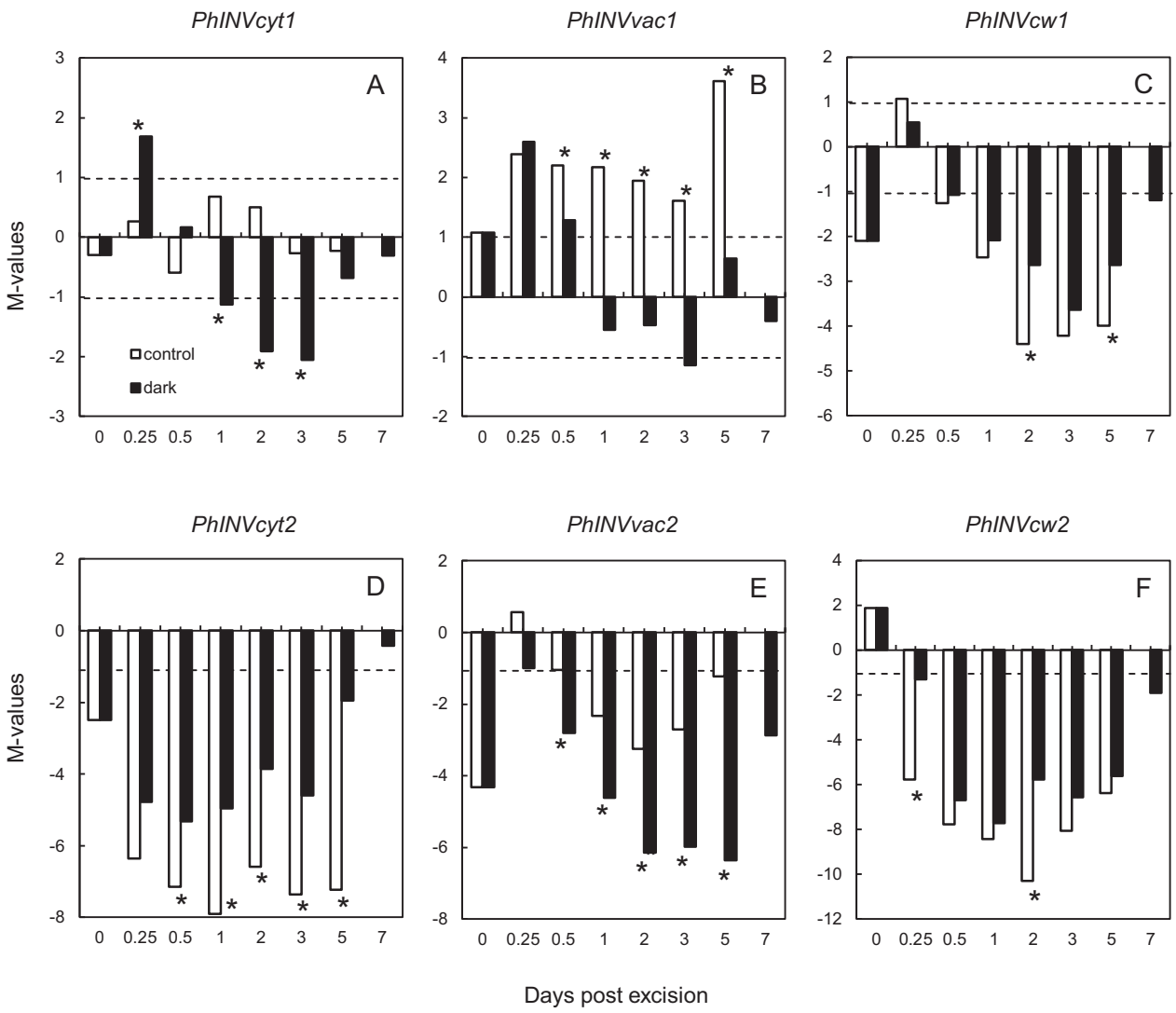

Fig. 7. Ratio of apex- to stem base-located RNA accumulation of genes encoding invertases during a period of 7 days after excision as affected by light conditions (illustrated in Fig. 1). M-value, $\log_2$ (RNA level-apex/RNA level-stem base). Mean values of two cytosolic (A, D: *PhINVcyt1*, *PhINVcyt2*), two vacuolar (B, E: *PhINVvac1*, *PhINVvac2*) and two cell wall (*PhINVcw1*, *PhINVcw2*) invertases are presented. Asterisks indicate significant differences between the control and the dark treatment at the specified time point (*t*-test, $P < 0.05$, $n = 3$, each sample from 6 to 9 individual cuttings).

and lower values were recorded for the dark exposed compared to the control cuttings (Figs. 5 and 6C, F). The INVcw gene *PhINVcw2* was expressed at very low levels in the apex but showed a strong up-regulation in the stem base of both control and dark exposed cuttings, peaking between 1 and 2 dpe (Figs. 6I, L). The maximum expression reached in dark exposed cuttings was about 40% of the maximum values attained in control cutting. Most strikingly, the pattern of *PhINVcw2* expression in the stem base of dark exposed and control cuttings between 0 and 1 dpe (Fig. 6L) mirrors the respective response profile of INVcw activity (Fig. 4H). After the dark treatment, the expression of *PhINVcw2* in the stem base remained on a continuously low level during the subsequent rooting under light.

The M-value calculated as $\log_2$ of the ratio of RNA levels between the shoot apex and the stem base highlighted a preferential expression of four INV genes in the rooting zone under control and dark conditions and a shift of the RNA accumulation ratios of three genes towards the rooting zone in response to the dark exposure (Fig. 7). Whereas the expression of the *PhINVcyt1* gene remained balanced between the shoot apex and the stem base under control conditions, current dark exposure from 1 dpe onwards shifted the RNA accumulation towards the rooting zone (Fig. 7A). In a similar way, dark exposure shifted the ratio of expression of *PhINVvac1* from a preferential apex-located expression to a balanced or even rooting zone-dominated expression (Fig. 7B). The genes *PhINVcyt2*, *PhINVvac2*, *PhINVcw1* and *PhINVcw2* were preferentially expressed in the stem base after excision under both control and dark conditions. In case of *PhINVcw2*, expression switched from an apex-dominated expression at harvest to a strong rooting-zone dominated expression while this effect was slightly more pronounced in control than in dark exposed cuttings (Fig. 7F). However, dark exposure promoted the rooting zone-dominated expression of the *PhINVvac2* gene compared to control cuttings (Fig 7E).

Planting and exposure of dark-treated cuttings to light again induced a preferential RNA accumulation of most of the genes in the rooting zone, except for *PhINVvac1* (Supplemental Fig. S1).

4. Discussion

Dark exposure of cuttings at reduced temperatures is an important step in production of more than one billion young ornamental plants per year in Europe. A better understanding of the molecular physiological processes involved should contribute to a better

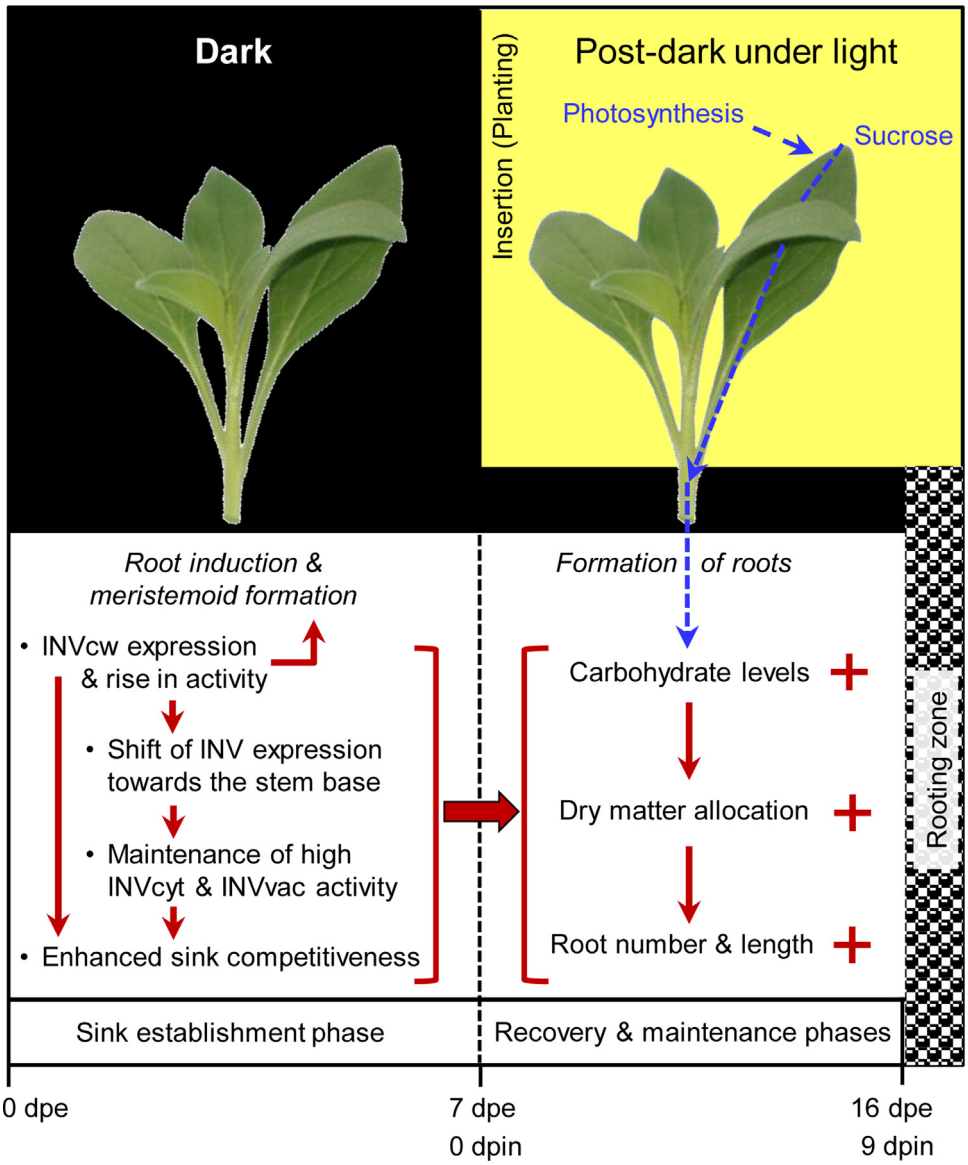

Fig. 8. Schematic presentation of the involvement of carbohydrate source-sink relationships in the dark stimulation of AR formation in petunia cuttings, based on the current study and on carbohydrate and rooting data published in Klopotek et al. [2]. Red arrows indicate stimulation. Red plus signs indicate promotive effects of dark pre-exposure compared to planting after excision.

understanding of plant plasticity and regeneration and open new perspectives for the fine tuning of production processes towards more efficient and sustainable propagation technologies. Based on recent studies [2] the question was addressed, how carbohydrate metabolism could be involved in dark-stimulated AR formation in petunia.

4.1. Role of photosynthesis and dry matter allocation in dark-stimulated AR formation

Taking into account that the observed carbohydrate depletion [2] may enhance photosynthesis via feed-forward control, we firstly investigated, whether dark exposure increases subsequent net photosynthesis during the rooting under light. The CO_2 gas exchange of cuttings was monitored in a recently developed open chamber system allowing online measurements of P_N and R_D on a whole cutting basis in the non-disturbed rooting environment [36]. Interestingly, both P_N and R_D were on the same levels for control and dark pre-exposed cuttings. Furthermore, the P_N levels were similarly as high as found for intact shoots still attached to the stock plant of the same cultivar [36]. These results clearly show that current P_N provides a substantial input of organic carbon into the cuttings, being not altered by dark pre-exposure. Contrary to our finding on petunia, the photosynthetic activity of *Impatiens hawkeri* cuttings was diminished by previous dark storage [41]. Photosynthesis of pelargonium cuttings was very low after dark storage when rooting under light and temperature conditions similar to those applied in the present study to petunia [21]. In the context of these studies, the present results indicate a high robustness of the photosynthetic machinery in *P. hybrida* 'Mitchell'. However, considering the strong leaf carbohydrate depletion of dark pre-exposed cuttings at time of planting [2], the photosynthetic activity is obviously not limited by feed-forward control under conditions as applied in the present study.

Since net carbon assimilation of cuttings is not altered by dark pre-exposure, we followed the hypothesis that the enhanced AR formation in dark treated cuttings is the outcome of selectively higher carbon allocation towards the developing root system in

the stem base. Considering the same time period after excision of cuttings, analyses of dry matter production of shoots versus roots clearly showed a higher carbon allocation to the rooting zone of dark treated cuttings than to that of control cuttings (Fig. 3C). The promoting effect of dark exposure on the carbon channelling towards the rooting zone became even more apparent, when control and dark pre-exposed cuttings experienced the same period of diurnal light after planting (Fig. 3D). Considering the total time period analysed, it became also apparent that the enhanced allocation of carbon to the rooting zone in the dark pre-treated cuttings was gained at the expense of reduced dry matter allocation to the shoot (Fig. 3B). This reduction was only marginally related to the generally high shoot dry matter production but nevertheless allowed a highly significant increase in root dry matter (Fig. 3D). Summarizing, the data suggest that dark exposure enhances the sink strength of the rooting zone against the upper shoot.

4.2. Involvement of invertase activity and expression

Considering the important role of invertases for building up sink strength [29–31], we monitored invertase activities and transcript levels of invertase-encoding genes in the rooting zone and in the shoot apex under the influence of dark exposure. It was assumed that the shoot apex is the most import utilization sink in shoot tip cuttings competing for carbohydrates with the rooting zone. Interestingly, maintenance of higher activities of INVcyt in both the apex and the basal stem under current darkness when compared to diurnal light conditions was associated with higher RNA levels of *PhINVcyt1* in both tissues (Figs. 4A, E and 5). However, the findings that the dark-stimulated RNA accumulation was more pronounced in the stem base than in the apex (Figs. 5 and 6A, D) and that the balance of expression was shifted towards the stem base by dark exposure (Fig. 7A) may indicate that this gene and the corresponding enzyme have a particular function for the carbohydrate sink in the rooting zone under darkness. Similarly, higher activities of INVvac were maintained in both organs under darkness but were accompanied by higher RNA accumulation levels of *PhINVvac1* and *PhINVvac2* in the stem base only (Fig. 5), shifting the balance of RNA accumulation of both genes towards the stem base (Fig. 7B, E). This may reflect important functions of INVvac in dark-mediated AR formation. Even though the particular function of INVvacs in young utilisation sinks is less clear [42], they control cell expansion by osmotic regulation [43]. It has to be considered here that the activity of invertases measured in the whole stem base does not reflect tissue-specific responses, which may be controlled by the individual isoforms.

Contrasting to the soluble invertases, the activity of INVcw showed a rooting zone-specific strong increase under both diurnal light and dark conditions (Fig. 4H). Furthermore, the early response pattern of activity showing a subdued but significant rise under darkness compared to control conditions was mirrored by the RNA accumulation of *PhINVcw2* (Fig. 6L). In addition, RNA accumulations of *PhINVcw1* accumulated under both dark and light conditions at 3 dpe, while much higher transcript levels were reached in the stem base than in the apex. These findings and the observation that the balance of RNA accumulation of both genes was shifted towards the rooting zone under both dark and control conditions strongly suggest that the early rise in activity and particularly *PhINVcw2* RNA accumulation might contribute to an early sink establishment occurring already during the dark period. Considering the already proven regulatory role of INVcw in development of plant organs [29,30], it can be assumed that the rise in RNA accumulation of INVcw-encoding genes and in corresponding enzyme activity contributes to the stimulation of cell division resulting in root meristemoid formation observed under darkness [2]. The sink strength of a meristem might originate from the extent of cell division [44]. Therefore, it is tempting to speculate that at the end of the dark period the high number of cell divisions in the developing root meristems drives the sink strength of the rooting zone.

4.3. Regulation of invertases

In the present study, the dark incubation of cuttings was applied in combination with reduced temperature of 10 °C. The reason is that dark incubation at higher temperature as applied to the controls causes yellowing and partial drying of leaves and production of unusual fine roots [2]. Since the promotive effect of dark exposure on root number and length is also observed with the higher temperature we consider darkness as the primary factor, but which is most effective in combination with the lower temperature because exhaustion of cuttings is avoided [2]. Nevertheless, also the lower temperature may have influenced the observed dark responses.

Invertases are regulated at transcriptional, post-transcriptional and post-translational level, while they are highly responsive to diverse environmental signals, particularly stress factors [42,43,45]. It has repeatedly been shown that the expression and activity of invertases is stimulated by light and reduced under darkness [46–48]. Some of these effects are based on a positive feed-forward mechanism, in which different invertases are metabolically induced by glucose or other sugars [43,45]. However, different family members of invertases show contrasting responses to a particular stimulus and the expression of the same gene can differ markedly in various tissues [43,49]. We have shown that during rooting under diurnal light sugars including glucose show a transient depletion in the rooting zone until 1 dpe and then remain on low levels until 3 dpe, while sugars decrease to even lower levels during 7 days under darkness [2]. Regarding the complexity discussed above it cannot be excluded that the changes in invertase expression and activity in response to excision and dark treatment may in part be mediated by the changed sugar levels.

The common principle, which influences the physiology of both control and dark-treated cuttings, is the excision of cuttings from the stock plant. This involves (1) wounding at the cutting site and (2) isolation from the functional integrity of the whole plant, which may lead to temporary depletion of water and minerals in the rooting zone [50]. Wounding has been shown to stimulate the expression of INVcw and INVvac encoding genes in roots and leaves of tomato [51–53]. Interestingly, wounding of leaves of the same petunia line as used in the present study, after 2 h induced the *PhINVcyt2* RNA accumulation, repressed *PhINVvac2*, but greatly induced *PhINVcw2* RNA accumulation by a factor of almost ten [32]. This supports the conclusion that wounding at the cutting site contributed to the induction of *PhINVcyt2* and *PhINVcw2* and also to the early repression of *PhINVvac2* in the rooting zone of both control and dark exposed cuttings. Obviously, the combination of darkness and low temperature attenuated both, the up-regulation of *PhINVcyt2* and *PhINVcw2* and the down-regulation of *PhINVvac2* (Fig. 5).

Water deficit in maize and wheat had mixed effects on transcript levels of invertases obviously depending on the particular tissue [49,54,55]. The dark treated cuttings were kept in polyethylene bags at reduced temperature without any watering. In contrast, control cuttings were watered daily but also exposed to other dehydration forces resulting from the light and higher temperature. Thus, we cannot exclude that different water relations after cutting excision have contributed to the observed responses in invertase expression and activity. It has been shown for bean plants that phosphate starvation can enhance the activities of INVcyt and INVcw in leaves and roots [56]. Early induction of specific phosphate transporters was observed in the rooting zone of petunia cuttings during rooting under light conditions [32]. This may reflect

P-deficiency, which may also have influenced the rooting zone specific expression and activity of invertases.

4.4. Integrating model

Based on the data-set presented, on recent studies [2] and on the basic metabolic concept of AR formation [31], a model on the involvement of carbohydrate source-sink relationships in the dark stimulation of AR formation in petunia cuttings is postulated (Fig. 8). During the dark phase, expression of INVcw-encoding genes, particularly of *PhINVcw2* in the stem base contributes to a local rise in INVcw activity, leading to conversion of sucrose to hexoses. These sugars regulate and feed the early cell division processes resulting in root meristemoids. The rise in INVcw activity contributes to the sink establishment in the rooting zone under darkness. The sink competitiveness of the rooting zone against the shoot is further enhanced by shifting the apex/stem base ratios of RNA accumulation of genes encoding INVcyts and INVvacs towards the stem base. There, this probably contributes to the maintenance of high activities of INVcyt and INVvac in particular tissues. The earlier finding, that during dark exposure starch and sucrose deplete in source leaves at 1 dpe already and that concurrently in the stem base high glucose and fructose levels are maintained at the expense of sucrose [2], supports the view that during the dark phase the invertases use local sucrose reserves and sucrose re-translocated from the leaves as substrate. At the end of the dark phase, the low activities of invertases indicate the end of the sink establishment phase. Then, the high cell division activity in the already established root meristemoids governs the sink strength. When dark pre-exposed cuttings are exposed to light, current photosynthesis provides synthesis of assimilates, which are partially channelled towards the rooting zone. This results in the replenishment of sugars, while a symplastic transport of sucrose provides the carbon influx (recovery and subsequent maintenance phase according to Ahkami et al. [31]). Based on the same time point after planting and start of light exposure of cuttings (dpin scale), the cell division driven higher sink competiveness of the rooting zone of dark pre-exposed cuttings contributes to enhanced carbon allocation to the rooting zone and root formation when compared to cuttings, which did not experience dark exposure before planting. This may be supported by a second increase in rooting zone-directed activity (Fig. 4G) and expression of particular invertase genes (Supplemental Fig. S1), probably as a result of light-mediated, sugar-induced feed-forward mechanisms.

Future studies should particularly focus on the functional role of the gene *PhINVcw2* in AR formation and its regulation by diverse factors. Taking into account that during rooting under light the activities of invertases in the rooting zone of petunia and of carnation cuttings were stimulated by polar auxin transport and by auxin application, respectively [57,58], and that improved rooting of *Eucalyptus* cuttings after far-red light treatment of donor plants was associated with concerted transcription of auxin- and carbohydrate homeostasis-related genes [59], future studies will also consider, whether the dark response of invertases and of AR formation in petunia cuttings is mediated by a changed auxin homeostasis and signalling.

Authors' contributions

Performed the experiments on photosynthesis and INV activity: Yvonne Klopotek. Performed the experiments on dry matter allocation and RNA accumulation of invertases: Uwe Druege. Annotated the invertase genes: Philipp Franken. Established the photosynthesis measuring system and edited the manuscript: Hans-Peter Klaering. Established the PCR protocols and tested the reference genes: Kerstin Fischer. Analyzed the data: Yvonne Klopotek, Uwe Druege. Developed the model: Uwe Druege. Wrote the paper: Uwe Druege, Yvonne Klopotek. Co-conceived the study and edited the manuscript: Bettina Hause, Mohammad R. Hajirezaei, Philipp Franken. Conceived the study and the experimental designs: Uwe Druege. Principal investigator: Uwe Druege.

Acknowledgements

We would like to thank Baerbel Broszies, Sabine Czekalla, and Ingo Hauschild, all of IGZ Erfurt and Großbeeren, for their skilful technical assistance. This work was funded by the Pakt für Forschung und Innovation of the Leibniz-Gemeinschaft, Germany (WGL) and by the Deutsche Forschungsgemeinschaft (DR 411/2-1) and was supported by the States of Brandenburg and Saxony-Anhalt, the Free State of Thuringia and the Federal Republic of Germany.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2015.11.001.

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